AD_____

Award Number: W81XWH-08-1-0647

TITLE: Thermally Targeted Delivery of a c-Myc Inhibitory Peptide in Vivo Using Elastin-Like Polypeptide

PRINCIPAL INVESTIGATOR: Dr. Gene Bidwell

CONTRACTING ORGANIZATION: University of Mississippi Medical Center
Jackson, MS 39216

REPORT DATE: October 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-10-2011 | Annual Summary | 2 SEP 2010 - 2 SEP 2011 |

**4. TITLE AND SUBTITLE**

Thermally Targeted Delivery of a c-Mic Inhibitory Peptide in Vivo Using Elastin-Like Polypeptide

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

W81XWH-08-1-0647

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Dr. Gene Bidwell

E-Mail: gbidwell@umc.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Mississippi Medical Center
Jackson, MS 39216

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Although surgical resection with adjuvant chemotherapy and/or radiotherapy are used to treat breast tumors, normal tissue tolerance, development of metastases, and inherent tumor resistance to radiation or chemotherapy can hinder a successful outcome. Therefore, it is necessary to consider alternative targeted therapeutic approaches for adjuvant therapy that would significantly reduce undesired side effects in normal tissues. This proposal describes a thermally responsive polypeptide (CPP-ELP-H1) that inhibits breast cancer cell proliferation by blocking the activity of the oncogenic protein c-Myc. The objective of the proposed research is to demonstrate that these genetically engineered polypeptides can be targeted to the tumor site by applying local hyperthermia and can inhibit tumor growth in an animal model. During the course of this training program, we have used fluorescently labeled polypeptides to demonstrate that thermal targeting can, in fact, be used to enhance the accumulation of an ELP-based c-Myc inhibitory polypeptide at the tumor site. We have defined the plasma clearance and biodistribution of the Bac-ELP1-H1 polypeptide, and we have used in vivo imaging to demonstrate tumor deposition and thermal targeting of the polypeptide in real time. We have also collected tumor reduction data using a lead c-Myc inhibitory polypeptide, and determined that treatment of mice with this polypeptide combined with thermal targeting resulted in a 70% inhibition of tumor volumes.

**15. SUBJECT TERMS**

Thermal targeting, drug delivery, Elastin-like polypeptide, c-Myc, breast cancer

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 21 | **19b. TELEPHONE NUMBER** *(include area code)* |

## Table of Contents

## Introduction

We have developed a thermally responsive polypeptide (CPP-ELP-H1) that inhibits c-Myc transcriptional activity and breast cancer cell proliferation (1). The objective of the proposed research is to demonstrate that after systemic administration *in vivo*, these genetically engineered polypeptides can be targeted to a breast tumor site by applying local hyperthermia and inhibit tumor growth. The amino acid sequence of the thermally responsive polypeptides is based on the elastin-like (ELP) biopolymer, which is soluble in aqueous solution below physiological temperature (37 °C), but aggregates when the temperature is raised above 41 °C (2). A cell-penetrating peptide (CPP), bactenecin (Bac), penetratin (Pen), or Tat, is conjugated to the ELP to enhance delivery of the polypeptide across the plasma membrane of the tumor cells. To the CPP-ELP is added a peptide derived from helix 1 (H1) of the helix-loop-helix domain of c-Myc (3), which inhibits transcriptional activation by c-Myc and consequently inhibits cancer cell proliferation (1, 4).

Our hypothesis is that intravenously delivered thermally responsive c-Myc inhibitory polypeptides are likely to be cleared under physiological conditions (37 °C). However, they will accumulate in breast tumors grown in mice, where externally induced local heat (40-42 °C) will be applied. The accumulated polypeptides will block c-Myc activity and consequently inhibit proliferation of the cancer cells. In order to address this hypothesis, the following specific aims were proposed: (1) To quantitate the *in vivo* distribution of CPP-ELP-H1 in normal and neoplastic tissue in an athymic tumor-bearing mouse model of breast cancer and (2) to evaluate the therapeutic efficacy of CPP-ELP-H1 in the treatment of neoplastic xenografts in the mammary tissue of athymic mice with and without localized hyperthermia. Successful completion of the proposed study will provide the first evidence that ELP can deliver a therapeutic molecule and reduce breast tumor size in a thermally targeted manner, and this work will obtain the necessary toxicity, pharmacokinetic, biodistribution, and efficacy data necessary to advance this technology toward the ultimate goal of human therapeutics. Specific targeting of the proposed therapeutic polypeptides to breast tumors by local hyperthermia would increase specificity and efficacy of treatment and reduce the cytotoxicity in normal tissues. Therefore, the proposed research may have a significant impact, leading this technology into clinical trials, and it may provide a powerful technology to treat and manage breast cancer.

## Body

### Training Program

Learning Animal Techniques. During the first year of this training program, much of my time was spent learning and practicing experimental techniques in animal models. These techniques include various surgeries, such as tumor implantation and arterial and venous catheter placement; intravenous and intraperitoneal drug administration; histological techniques, including cutting tissue sections with a cryomicrotome and performing various staining techniques (H&E and immunostaining); and data collection and analysis, including quantitative analysis of autoradiograms and fluorescence images. These techniques have been and will be invaluable for my future career as an independent breast cancer researcher. Without these tools, I would not be able to effectively carry out drug development and testing in animal models.

Coursework. As outlined in the original proposal, I successfully completed a histology course taught by my Co-mentor Dr. Hamed Benghuzzi. This course taught me the basics of preparing tissue for sectioning and identifying various tissues and cell types within them. Dr. Benghuzzi continues to mentor me to develop my knowledge of histology and to be able to use these techniques to identify signs of toxicity or cellular stress.

Lecture Series and Collaboration. In addition to the training and coursework listed above, I have also continually attended lectures pertinent to breast cancer by guest speakers. These lectures have covered multiple topics, including Notch signaling in breast cancer, the role of obesity in breast cancer risk, microRNAs and breast cancer, RGS protein signaling in breast cancer, and the role of the BRCA1-IRIS protein in breast cancer development. Our Cancer Institute at the University of Mississippi Medical Center has many active breast cancer researchers, which provides the opportunity to interact and exchange ideas with leading scientists in the field. One example of such interaction includes my participation, via a collaboration between my mentor Dr. Drazen Raucher and our Cancer Institute's Director, Dr. Lucio Miele, in a project to develop and test novel Notch I inhibitors for breast cancer therapy.

Teaching. In addition to the focused course work and lectures, I have also learned details of breast cancer development, characterization, and therapy by teaching a series of lectures to the Department of Biochemistry's graduate students. During these lectures, I covered topics of breast cancer development, molecular and histological classification, and existing and upcoming therapeutics for breast cancer treatment. I have also served as a mentor to incoming graduate students, teaching them the basics of drug delivery techniques, the hurdles of breast cancer therapy, and the use of animal models to test new approaches for breast cancer treatment.

Mentorship. I continue to work daily with my Co-mentor, Dr. Drazen Raucher, who oversees all aspects of the drug development and testing. I meet regularly with Dr. Benghuzzi, who, in addition to the hands on instruction described above, also follows my progress on the proposed research with helpful advice. In addition to mentorship by Dr. Raucher and Dr. Benghuzzi, I have also enjoyed the benefit of instruction in other animal surgeries by Dr. Eddie Perkins, Assistant Professor of Neurosurgery at UMMC, who taught me to administer glioma cells to develop brain tumors in a rat model of glioblastoma and to monitor these tumors by MRI scanning; and by Dr. David Stec, Assistant Professor of Physiology at UMMC, who taught me to place indwelling venous catheters in the jugular vein of mice and to tunnel the catheters out the back of the animal's neck for repeated administration of compounds via the venous route. I also attended the 2009, 2010, and 2011 LINKS meetings in Washington, DC, and the 2011 Era of Hope meeting in Orlando, FL, which were unique opportunities to interact with other very bright postdoctoral fellows and with highly distinguished leaders in breast cancer research. I gained an enormous amount of knowledge from this meeting, not only specific to my research, but also about the state of breast cancer research in general and the areas of new development in the field.

### *In vitro* Optimization of a Lead Polypeptide Construct

Before beginning *in vivo* analysis, we optimized the intracellular delivery of the ELP-fused c-Myc inhibitory peptide *in vitro*. In order to enhance uptake of ELP-based polypeptides, we fused them with cell penetrating peptides (CPPs). CPPs are short peptides known to enhance the cellular uptake of large cargo. The three CPPs proposed for this study are the penetratin (Pen) peptide from Antennapedia (5), the Tat peptide from the HIV-1 Tat protein (6), and the Bac peptide from the bovine antimicrobial bactenecin peptide Bac 7 (7). We proposed to use these CPPs for delivery of the thermally responsive ELP polypeptide fused to a peptide inhibitor of c-Myc. Previously, we conjugated the c-Myc inhibitory peptide and the Pen peptide to ELP for thermally targeted delivery (Pen-ELP-H1) (1). Uptake of Pen-ELP-H1 in MCF-7 cells was increased by both the Pen peptide and by the hyperthermia-induced aggregation of ELP. It was demonstrated that Pen-ELP-H1 could sequester endogenous c-Myc to the cytoplasm, thus preventing its interaction with Max or other nuclear

partners and its activation of transcription. The result of this inhibition was reduction in the proliferation rate of the MCF-7 cells. In addition, Pen-ELP-H1 increased the potency of topoisomerase II inhibitors, demonstrating potential utility for combination therapy (8). However, after a short exposure to Pen-ELP-H1, inhibition of cell proliferation was only observed 11 days later. In an attempt to increase the potency of the ELP-H1 polypeptide before beginning *in vivo* testing, it was fused to the Tat or Bac CPPs. We determined the cellular uptake, subcellular distribution, and proliferation inhibition for each construct. The results of this *in vitro* testing were published (9). To summarize the major findings, all three CPP-ELP-H1 constructs were capable of inhibiting the proliferation of MCF-7 breast cancer cells. Of the three CPPs, the Pen peptide delivered the most cargo into the cells, but the Bac-ELP-H1 polypeptide was the most potent inhibitor of breast cancer cell proliferation. It was determined that the Bac peptide was capable of delivering the ELP-H1 polypeptide into the nucleus of some of the target cells, whereas Tat and Pen both delivered the cargo polypeptide into the cytoplasm only. We concluded that, although the total cellular uptake was lower for Bac-ELP-H1, its ability to reach the nucleus, and likely interfere with the c-Myc/Max interaction directly, made this construct more potent than the Pen or Tat – containing constructs. Therefore, we elected to use Bac-ELP-H1 as the lead polypeptide for *in vivo* testing.

**Summary of Progress in Years 1 and 2.** During the first year of work on the proposed studies, we tested the three proposed CPP-ELP-H1 polypeptides *in vitro*, and identified Bac-ELP-H1 as a lead peptide for *in vivo* testing. A biodistribution experiment was carried out in nude mice bearing MCF-7 tumors. Plasma clearance curves were determined following both IV and IP administration of radio-labeled Bac-ELP-H1, and tumor and organ uptake were defined by autoradiography. Application of hyperthermia lead to a 1.5 fold enhancement of tumor polypeptide levels relative to the unheated tumor. This experiment elucidated three areas where improvement to the experimental methods were necessary. The use of $^{125}$I to label the protein was found to be problematic because the label was not stable in circulation. An alternative method using fluorescently labeled polypeptides was outlined. This method has been used successfully to carry out the experiments of Aim 1, as shown below. Secondly, in response to recent literature and optimization in our own lab, the tumor heating protocol was changed from 1 hour of continuous heating to a heat cycling protocol in which tumors are heated for four twenty minute cycles separated by ten minute cooling periods. Finally, following the suggestions of several senior researchers at the 2009 LINKS meeting, the animal model for initial investigation of polypeptide efficacy was changed from an MCF-7 xenograft model to a syngeneic, orthotopic model. This model, which utilizes mouse E0771 breast cancer cells growing in the mammary fat pad of C57BL/6 mice, allows evaluation of therapeutic efficacy in an animal with an intact immune system. Effects of immunogenicity of the test agent on its efficacy or its induction of adverse events can be elucidated using this model that would be missed using immunocompromised xenograft models.

During the second year of funding, we performed biodistribution and tumor reduction studies with the lead Bac-ELP-H1 agent in the E0771 breast tumor model. At the time the last progress report was submitted, we had nearly completed the biodistribution study, and we lacked some control experiments in the tumor reduction study. During the third year of funding, all biodistribution and tumor reduction studies have been completed, and the results are described below. Also, this data has been submitted for publication, and, at the time of this writing, the manuscript is under consideration at *Science Translational Medicine.*

**Specific Aim 1: Polypeptide Biodistribution and Pharmacokinetics**

Specific Aim 1 of the proposal is to quantitate the *in vivo* distribution of CPP-ELP-H1 in normal and neoplastic tissue in a mouse model of breast cancer. We have chosen to use E0771 breast cancer cells implanted in the mammary fat pad of C57BL/6 mice. Both the IV (intravenous) and IP (intraperitoneal) routes of administration are being investigated

Heating Breast Tumors Using Infrared Light. To apply hyperthermia to rodent tumors, we illuminate them with infrared light (IR, 950 nm) generated from the SLD/LED cluster of a Laser Sys-Stim 540 (Mettler Electronics, Anaheim, CA). This device allows penetration of IR light up to 4 mm deep in tissues, and the light source can be applied directly over the tumor site while shielding surrounding areas from illumination, providing a focused source of hyperthermia. A previous study demonstrated that ELP deposition in tumor is enhanced by cycling between periods of mild hyperthermia and cooling periods (10). Mild hyperthermia (40 – 42 $^0$C) causes ELP to aggregate and accumulate in the tumor vasculature, and upon return to normothermia, ELP re-dissolves and diffuses down the concentration gradient that has formed from the vasculature into the extravascular space. As shown in Figure 1, the core temperature in the tumor can be raised from 33 $^0$C to 41 $^0$C within 20 min by applying IR light. When the light source is removed, the tumor core temperature returns to

body temperature very quickly. Thermal cycling between the desired hyperthermia temperature of 40 – 42 $^0$C and body temperature can be achieved repeatedly with 20 min cycles of IR illumination, followed by 10 min cooling periods. During this process, the mouse's body temperature does not rise above 37 $^0$C, and the skin over the tumor site is not significantly heated by the IR light, presumably because absorbance of the light over some depth is required for the temperature to increase. This schedule of 4 20 min heating cycles followed by 10 min cooling cycles is used for hyperthermia application throughout this study.

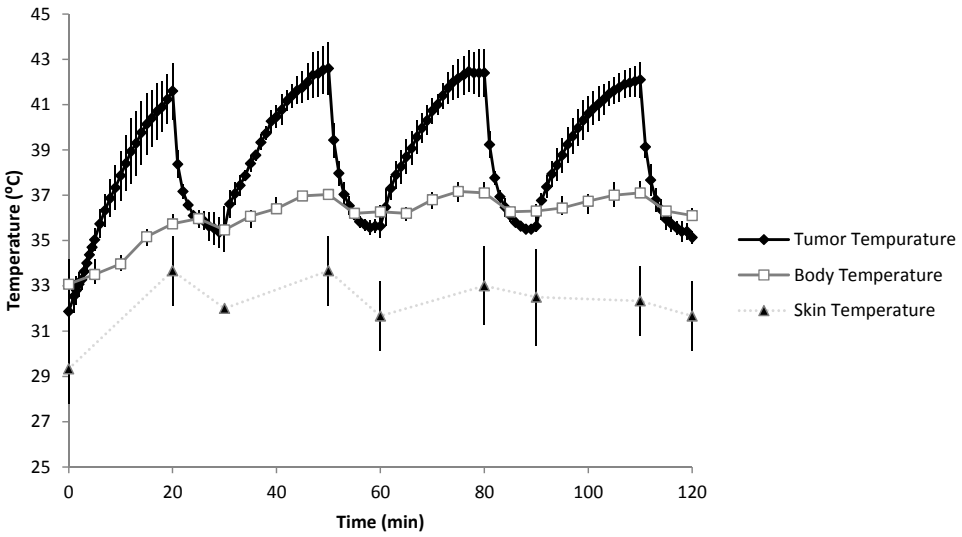



**Figure 1. Heating Tumors with Infrared Light.** Tumor temperature (as monitored by a needle thermocouple in the tumor core), body temperature, and skin temperature over the heated site was recorded while illuminating the tumor with 950 nm light from an LED light source. Data represent the mean of three mice bearing 250 mm$^3$ E0771 mammary tumors, bars, s.d.

Pharmacokinetics of the Bac-ELP – delivered c-Myc Inhibitory Peptide. Plasma levels and tumor and organ biodistribution were examined after administration of rhodamine-labeled Bac-ELP-H1. Both the intravenous (IV) and intraperitoneal (IP) routes were examined. Following IV injection, the thermally responsive Bac-ELP1-H1 polypeptide was cleared from circulation (Figure 2A), and the data were well described by a two-compartment pharmacokinetic model. The terminal half-life was found to be 102.0 ± 9.2 min and the plasma AUC was 9,713.5 ± 1,393.9 μg·min/mL (Table 1). When the tumor was heated for 2 h after injection using the heat cycling protocol described above, the distribution half-life was slightly faster, but the difference was not statistically significant. The terminal half-life was unaffected by tumor hyperthermia. (Table 1). The non-thermally responsive Bac-ELP2-H1 polypeptide had a slightly slower terminal half life and a lower initial plasma concentration than Bac-ELP1-H1, and, as expected due to its lack of aggregation, tumor hyperthermia had no effect on the pharmacokinetic parameters of Bac-ELP2-H1 (Table 1). Following IP administration, the polypeptide slowly entered systemic circulation. 90 min after injection, the plasma level of Bac-ELP-H1 was equal to that observed after IV injection, and the plasma levels peaked at 150 min post injection (Figure 2A). These data demonstrate that the IP route leads to equivalent plasma levels as obtained by IV injection within approximately one half-life, and the IP route may be a viable method for administering multiple chronic doses.

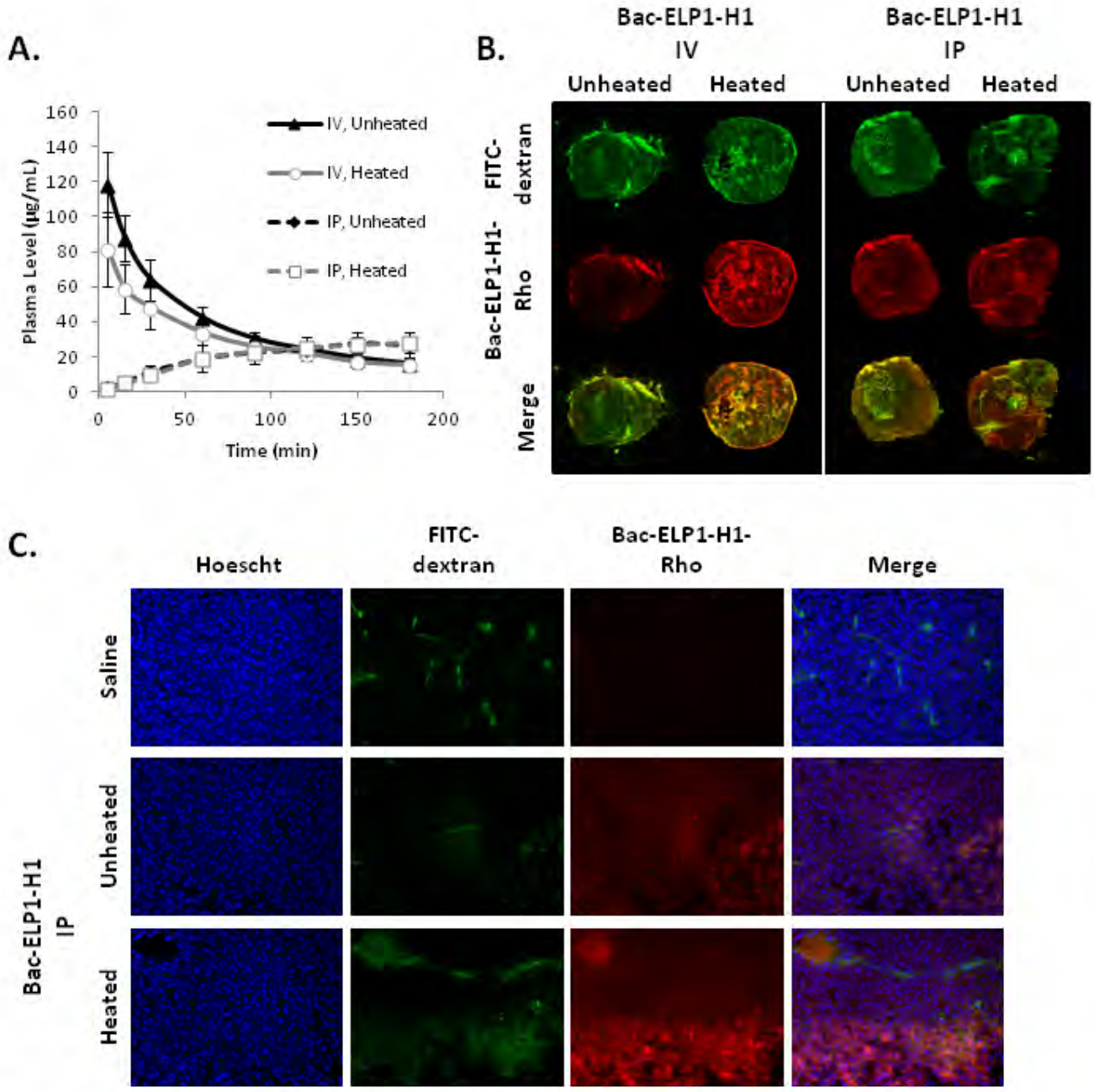



**Figure 2. Plasma Clearance and Tumor Uptake of Bac-ELP-H1.** **A.** Plasma levels with time following IV or IP injection of Bac-ELP1-H1. Data represent the mean ± s.d. of 6 animals per group. **B.** Representative images of tumor sections 3 h after IV or IP injection of rhodamine-labeled Bac-ELP1-H1. The perfused vasculature was marked by infusion of high molecular weight dextran 1 min prior to euthanasia (top panel), and rhodamine fluorescence was used to follow the localization of the polypeptide within the tumor (middle panel). **C.** High magnification images of the tumors in **B** were obtained using a fluorescence microscope and a 40 x objective. Cell nuclei were stained with Hoescht 33342.

| | | | Bac-ELP1-H1 (Tumor Unheated) | Bac-ELP1-H1 (Tumor Heated) | Bac-ELP2-H1 (Tumor Unheated) | Bac-ELP2-H1 (Tumor Heated) |
|---|---|---|---|---|---|---|
| Initial Concentration | $C_o$ | (ug/mL) | 140.2 ± 28.9 | 95.0 ± 26.7 | 78.9 ± 7.5 | 81.7 ± 12.5 |
| Central Compartment Volume of Distribution | $V_c$ | (mL) | 11.1 ± 1.7 | 15.5 ± 5.3 | 20.2 ± 2.3 | 23.2 ± 3.1 |
| Plasma Clearance | $Cl$ | ($mL \cdot min^{-1}$) | 0.16 ± 0.03 | 0.18 ± 0.05 | 0.28 ± 0.04 | 0.32 ± 0.06 |
| Area Under Curve | $AUC$ | ($\mu g \cdot min \cdot mL^{-1}$) | 9,713.5 ± 1,393.9 | 7,752.4 ± 1,665.6 | 5,786.1 ± 861.5 | 6,104.4 ± 1,135.3 |
| Tissue to Plasma rate constant | $k_{tp}$ | ($min^{-1}$) | 0.025 ± 0.008 | 0.047 ± 0.019 | 0.029 ± 0.004 | 0.026 ± 0.004 |
| Plasma to Tissue rate constant | $k_{pt}$ | ($min^{-1}$) | 0.021 ± 0.014 | 0.032 ± 0.014 | 0.049 ± 0.017 | 0.034 ± 0.009 |
| Elimination rate constant | $k_{el}$ | ($min^{-1}$) | 0.014 ± 0.003 | 0.012 ± 0.002 | 0.014 ± 0.001 | 0.014 ± 0.002 |
| Distribution Half Life | $t_{1/2,dist}$ | (min) | 14.6 ± 4.5 | 9.4 ± 3.4 | 8.4 ± 2.0 | 10.4 ± 1.9 |
| Terminal Half Life | $t_{1/2,term}$ | (min) | 102.0 ± 9.2 | 104.4 ± 15.2 | 152.7 ± 28.7 | 136.9 ± 23.0 |

**Table 1. Plasma Pharmacokinetics of Bac-ELP-H1.**

Tumor Accumulation and Biodistribution of Bac-ELP-H1. Tumor and organ biodistribution was determined 3 h after IV or IP administration of rhodamine-labeled Bac-ELP-H1 by quantitative fluorescence analysis of cryo-sections using a slide scanner. As shown in Figure 2B, Bac-ELP1-H1 accumulated to detectable levels in tumor following either IV or IP administration, and tumor levels of the polypeptide were visibly higher in hyperthermia treated tumors. Tumor levels of the thermally responsive Bac-ELP1-H1 polypeptide trended higher by 1.57 fold and 1.52 fold with hyperthermia treatment following injection by the IV and IP routes, respectively (Figure 3A). Both administration routes resulted in similar tumor levels. A non-thermally responsive control polypeptide, Bac-ELP2-H1, was used to determine whether the enhanced tumor deposition was due to polypeptide aggregation or to non-specific effects of hyperthermia. Bac-ELP2-H1 has a similar composition and molecular weight to the active Bac-ELP1-H1 polypeptide, but it does not aggregate at the mild hyperthermia temperatures used in this study (11). Hyperthermia treatment did not cause an increase in the tumor levels of Bac-ELP2-H1 (Figure 3B), indicating that the thermal targeting observed with Bac-ELP1-H1 is due to the polypeptide's hyperthermia-induced aggregation. Enhanced polypeptide levels in heated tumors is promising, but in order for thermal targeting to be effective, the polypeptide must escape the vasculature, traverse the extracellular matrix of the tumor, and enter the tumor cells. We used an IV infusion of 500 kDa FITC-dextran 1 min prior to euthanasia to mark the perfused vasculature of the tumor. Figure 2B shows that Bac-ELP1-H1 is not confined to the vascular space. Higher magnification images, using Hoescht 33342 to mark cell nuclei, demonstrate that Bac-ELP1-H1 is present in the tumor cells as well as in the blood vessels (Figure 2C).

Biodistribution of Bac-ELP1-H1 in the major organs was also determined after IV or IP injection by quantitative fluorescence analysis (Figure 3C and Figure 4). Bac-ELP1-H1 was detectable in all major organs over background fluorescence following both injections routes, with the exception of the brain following IV injection (Figure 4A and 4B). In most organs, the polypeptide was uniformly distributed and localized very close to the perfused vasculature. The only exception was in the kidney, where the polypeptide was primarily concentrated in the renal cortex. The polypeptide accumulated to high levels in the liver and kidney, and much lower levels were detected in the heart, spleen, lungs, and brain. IP administration led to much lower accumulation in the liver relative to the IV route ($p = 0.0006$, one-way ANOVA, post hoc Bonferroni), spleen levels were higher following IP injection compared to IV injection ($p = 0.0005$), and brain levels were

undetectable over background fluorescence following IV injection. Tumor hyperthermia did not significantly affect polypeptide levels in any organ except the spleen.

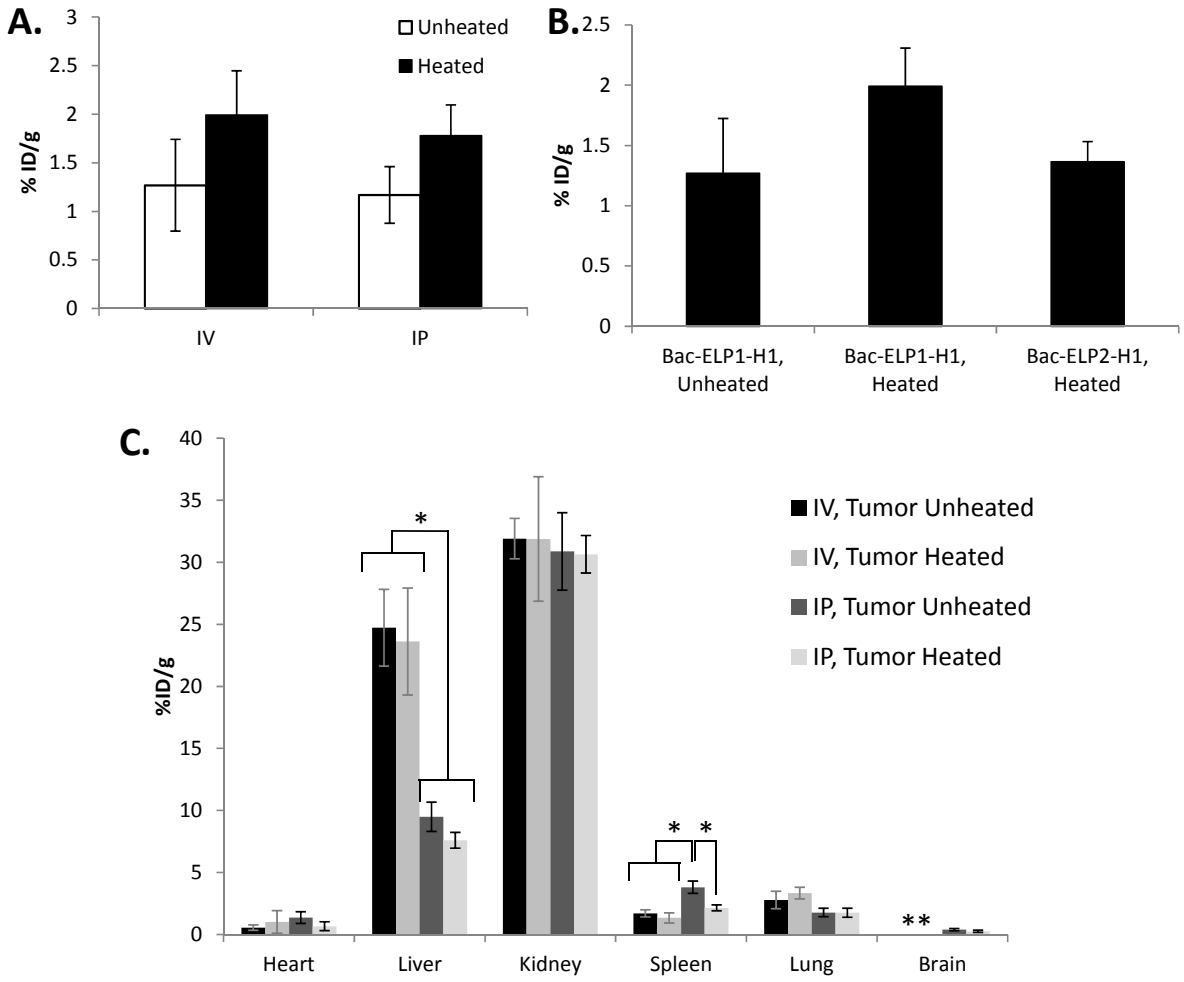
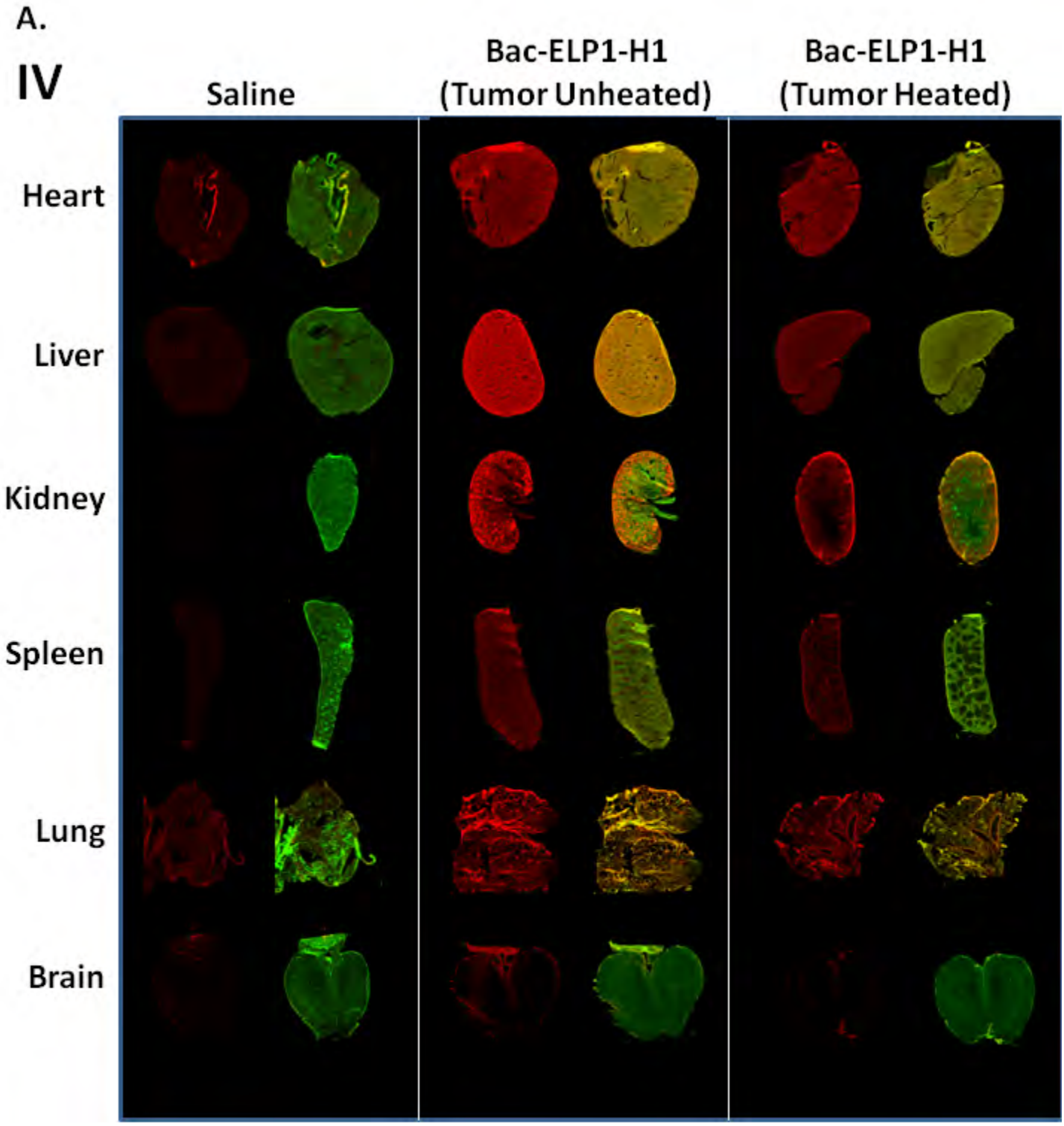



**Figure 3. Biodistribution of Bac-ELP-H1 Following IV or IP Injection. A.** Bac-ELP1-H1 tumor levels 3 h after IV or IP administration of rhodamine-labeled Bac-ELP1-H1 with and without tumor hyperthermia. **B.** Bac-ELP1-H1 and Bac-ELP2-H1 tumor levels 3 h after IV administration of polypeptides with or without hyperthermia. Bars, s.e. **c,** Organ distribution of Bac-ELP1-H1 3 h after IV or IP administration with or without tumor heating. Bars, s.e. $^{*}$, Organ levels are statistically different, (one-way ANOVA, p = 0.0006 (liver), p = 0.005, (spleen), post hoc Bonferroni, 95% CI). $^{**}$ Brain levels were not detectable over background fluorescence following IV injection.


A.
IV
Saline
Bac-ELP1-H1
(Tumor Unheated)
Bac-ELP1-H1
(Tumor Heated)
Heart
Liver
Kidney
Spleen
Lung
Brain

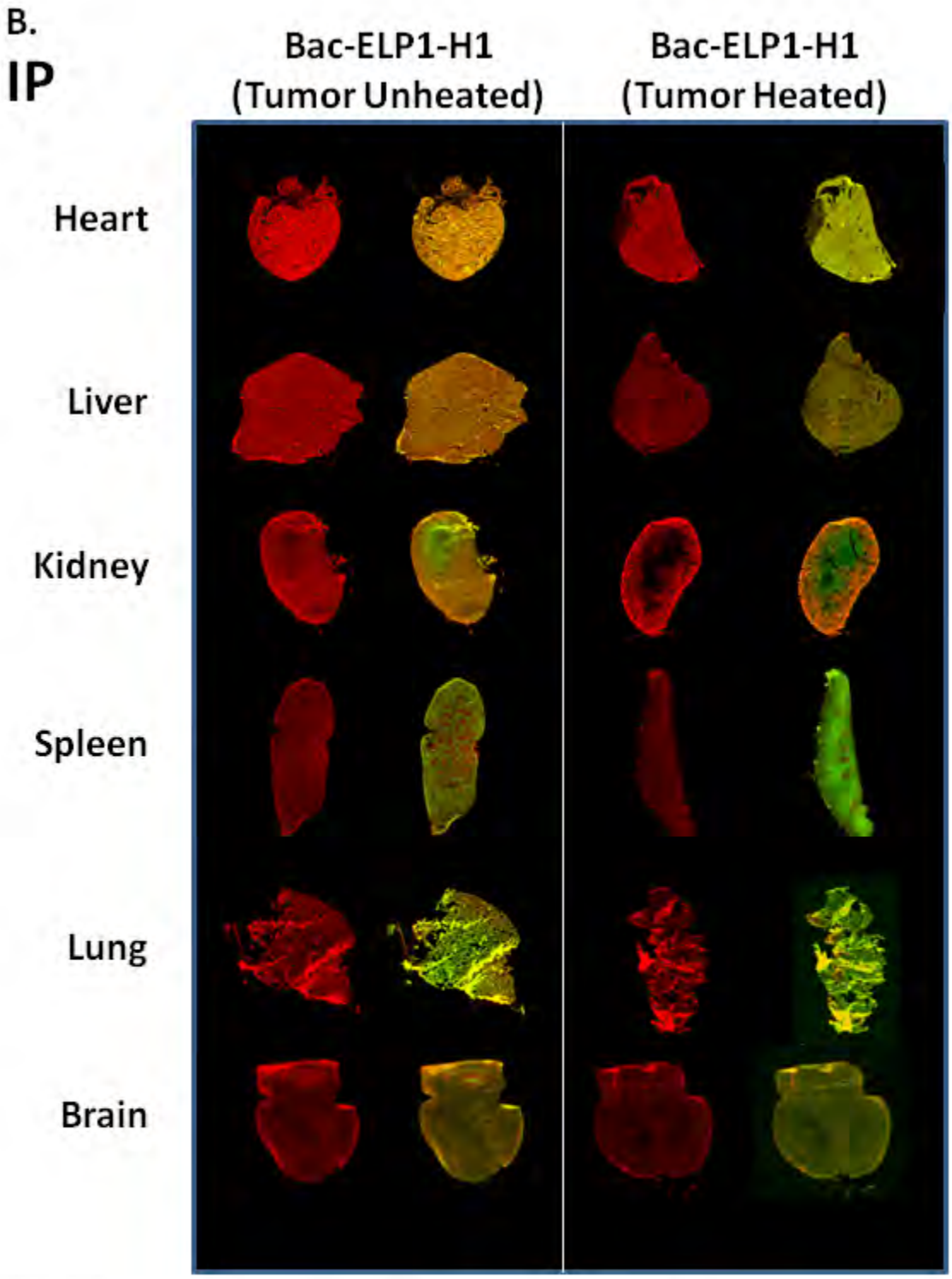



**Figure 4. Organ Fluorescence Following IV or IP Injection.** Representative sections of each organ are shown follow IV (**A**) or IP (**B**) injection of rhodamine-labeled Bac-ELP1-H1 or saline control. PMT values were adjusted between scans of different organs, so the image intensity from one organ to the next is not representative of the difference in polypeptide levels.

Accumulation of Bac-ELP-H1 in the Tumor in Real Time Using *in vivo* Imaging. We used *in vivo* fluorescence imaging to follow polypeptide levels in tumors longitudinally after IV injection. The polypeptide was labeled with Alexa Fluor-750. The infrared fluorophor was chosen due to the low tissue autofluorescence at these wavelengths. The polypeptide was injected IV in both tumor bearing and non-tumor bearing mice, and the tumor mice were divided into unheated and hyperthermia-treated groups. Images were collected at 2, 3, 4, 6, 8, 24, 48, and 72 h after injection. Bac-ELP1-H1, labeled with Alexa 750, could be detected in the tumors for up to 48 h after administration (Figure 5). As shown in Figure 6A, Bac-ELP1-H1 accumulated to the highest levels in the tumor, liver, and bladder. Quantitation of the tumor levels, corrected for background fluorescence from non-tumor mammary tissue, indicated that Bac-ELP1-H1 deposition peaked in tumors 6 h after injection, and thermal targeting resulted in as much as 2.8 fold more Bac-ELP1-H1 in the heated tumor than in the unheated tumor (Figure 6B, $p = 0.007$, student's t-test).

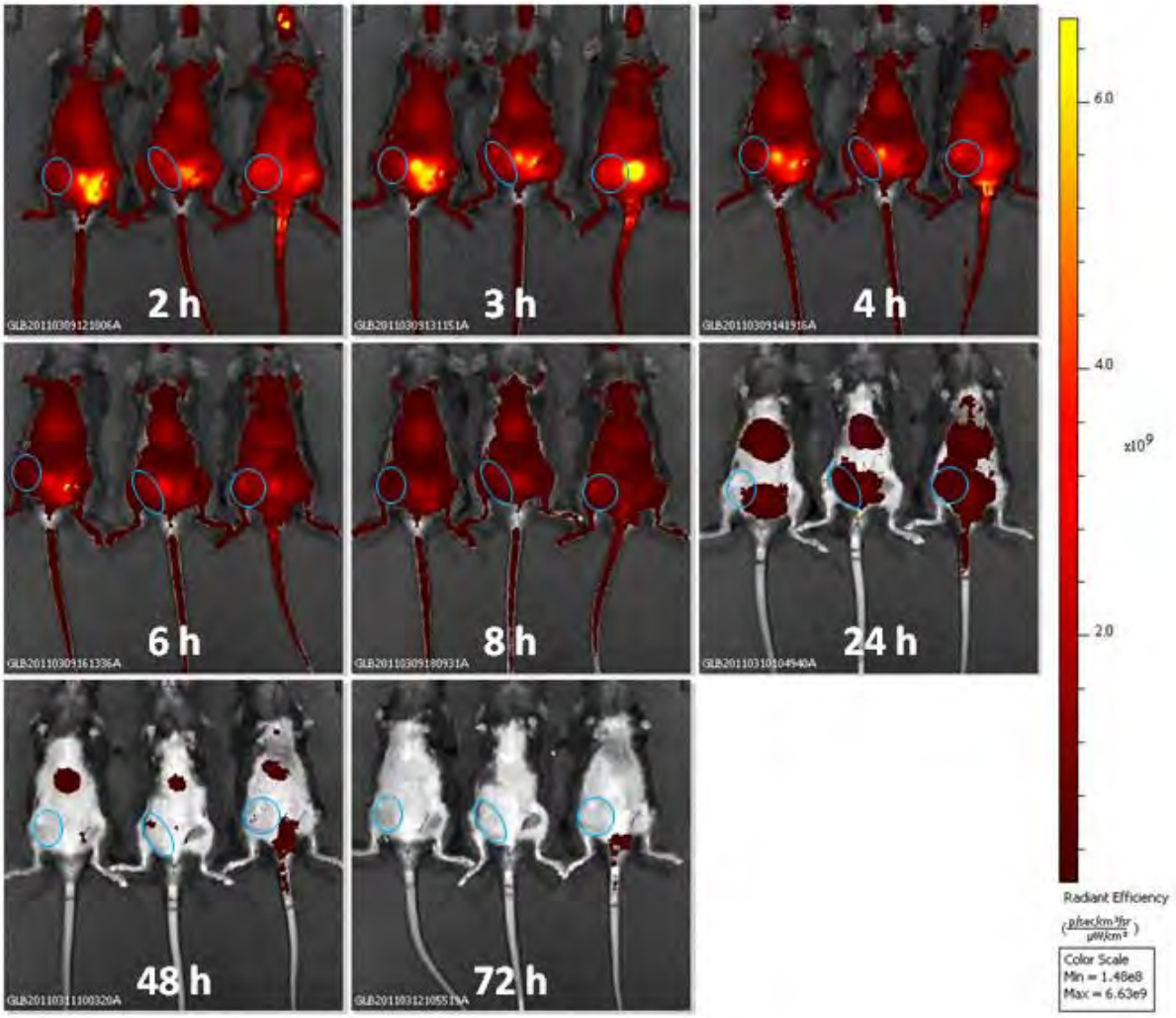



**Figure 5. *In vivo* Fluorescence Images of Bac-ELP-H1 Tumor Deposition over Time.** Images at various time points after IV administration of Alexa Fluor 750-labeled Bac-ELP1-H1 are shown. The left animal has no tumor, in the center animal, the tumor was unheated, and in the right animal, the tumor was heated. The position of the tumor or corresponding non-tumor bearing mammary fat pad are shown by the ovals.

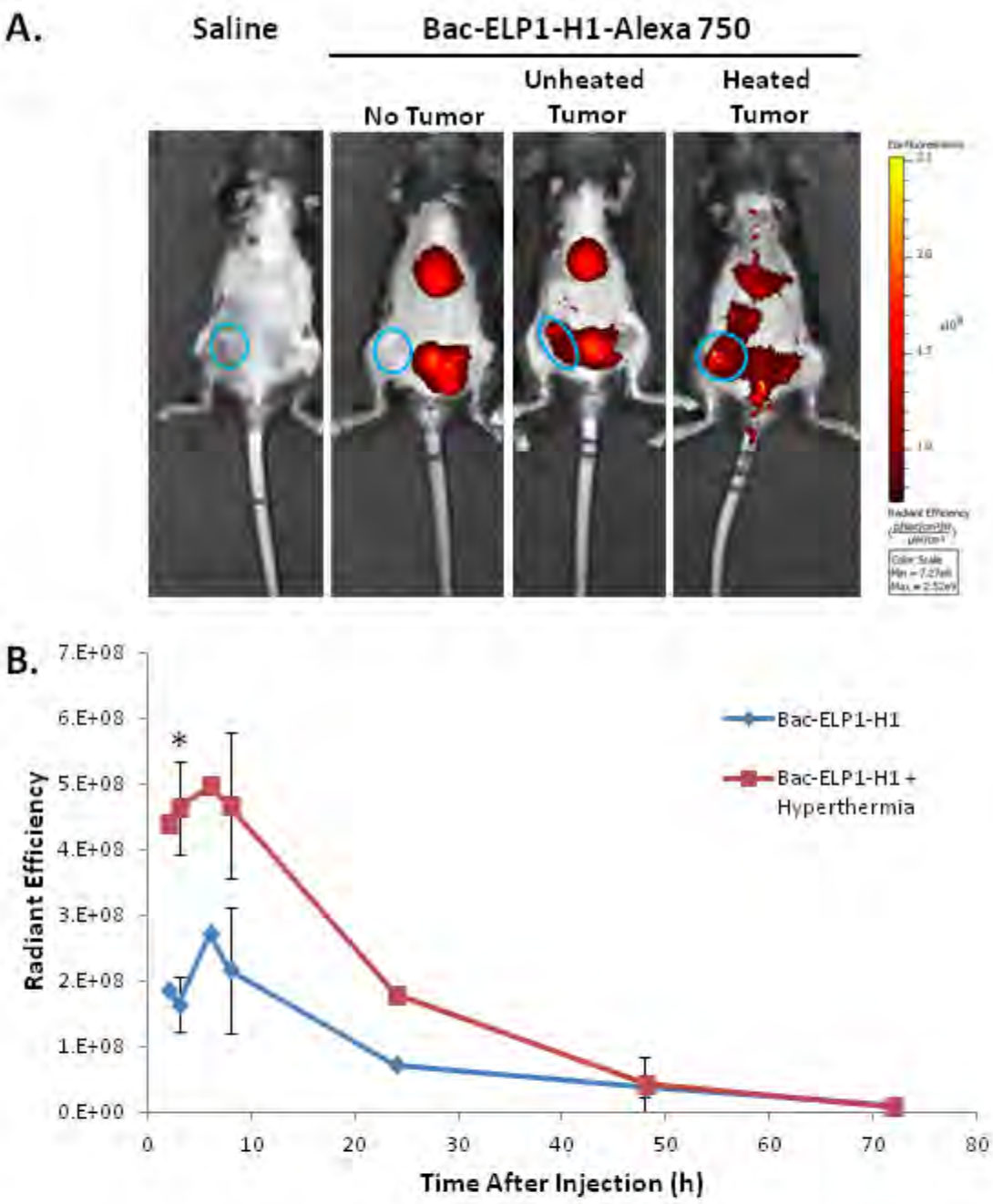



**Figure 6.** ***In vivo*** **Tumor Deposition of Bac-ELP-H1 with Time.** **A.** Representative fluorescence images 8 h after injection with saline (left panel) or Alexa Fluor 750-labeled Bac-ELP1-H1 (right panels). A mouse with no tumor (second panel), an unheated tumor (third panel), and a heated tumor (fourth panel) are shown. The position of the tumor or the corresponding non-tumor bearing mammary fat pad are shown by the ovals. **B.** Quantitative analysis of the tumor fluorescence at each time point. Data represent the mean ± s.e. of 5 mice / group. Alternating error bars omitted for clarity. *, The difference between heated and unheated tumors is statistically significant ($p = 0.007$, student's t-test).

**Specific Aim 2: Tumor Size Reduction**

Specific Aim 2 of the proposal is to evaluate the therapeutic efficacy of CPP-ELP-H1 in the treatment of tumors in the mammary tissue of mice with and without localized hyperthermia. Repeated injections of the lead polypeptide from Specific Aim 1 or different control polypeptides, coupled with local hyperthermia, were proposed to be compared for their effect on the tumor size.

Inhibition of Tumor Progression by Bac-ELP-H1. Having demonstrated that thermal targeting results in significantly enhanced levels of Bac-ELP1-H1 in tumors, we tested the ability of the polypeptide, with and without hyperthermia, to reduce tumor growth. Polypeptide injections were initiated when the E0771 tumors reached 150 $mm^3$, and, based on the pharmacokinetic and biodistribution results, polypeptides were given daily at a dose of 200 mg/kg/day via IP administration. As shown in Figure 7A, the control E0771 tumors reached an average volume of 2500 $mm^3$ within 14 days of the start of treatment. Hyperthermia alone, or the control polypeptide Bac-ELP1 (which lacks the c-Myc inhibitory peptide), had no effect on tumor volume. Bac-ELP1-H1, when given daily for 7 days without hyperthermia, caused a slight reduction in the tumor growth rate, but the results were not statistically significant. However, when Bac-ELP1-H1 treatment was combined with hyperthermia, the tumor volumes were reduced by nearly 70% ($p = 0.0017$,one-way ANOVA, post hoc Bonferroni). All polypeptides caused some body weight loss in the mice during the treatment period (Figure 7B and D), but the body weight quickly recovered after removal of the treatment, and the mice showed no other signs of toxicity. The non-thermally responsive control polypeptide Bac-ELP2-H1 reduced tumor growth in a similar manner to that observed from Bac-ELP1-H1 treatment without hyperthermia, and addition of hyperthermia had no effect on the antiproliferative effect of Bac-ELP2-H1 (Figure 7C). These data are consistent with the tumor uptake data, and indicate that the enhanced anti-tumor effect seen with Bac-ELP1-H1 + hyperthermia is due to thermal targeting of the polypeptide. The tumor volume results were confirmed by measuring the tumor mass at necropsy on day 14 after treatment (Figure 7E).

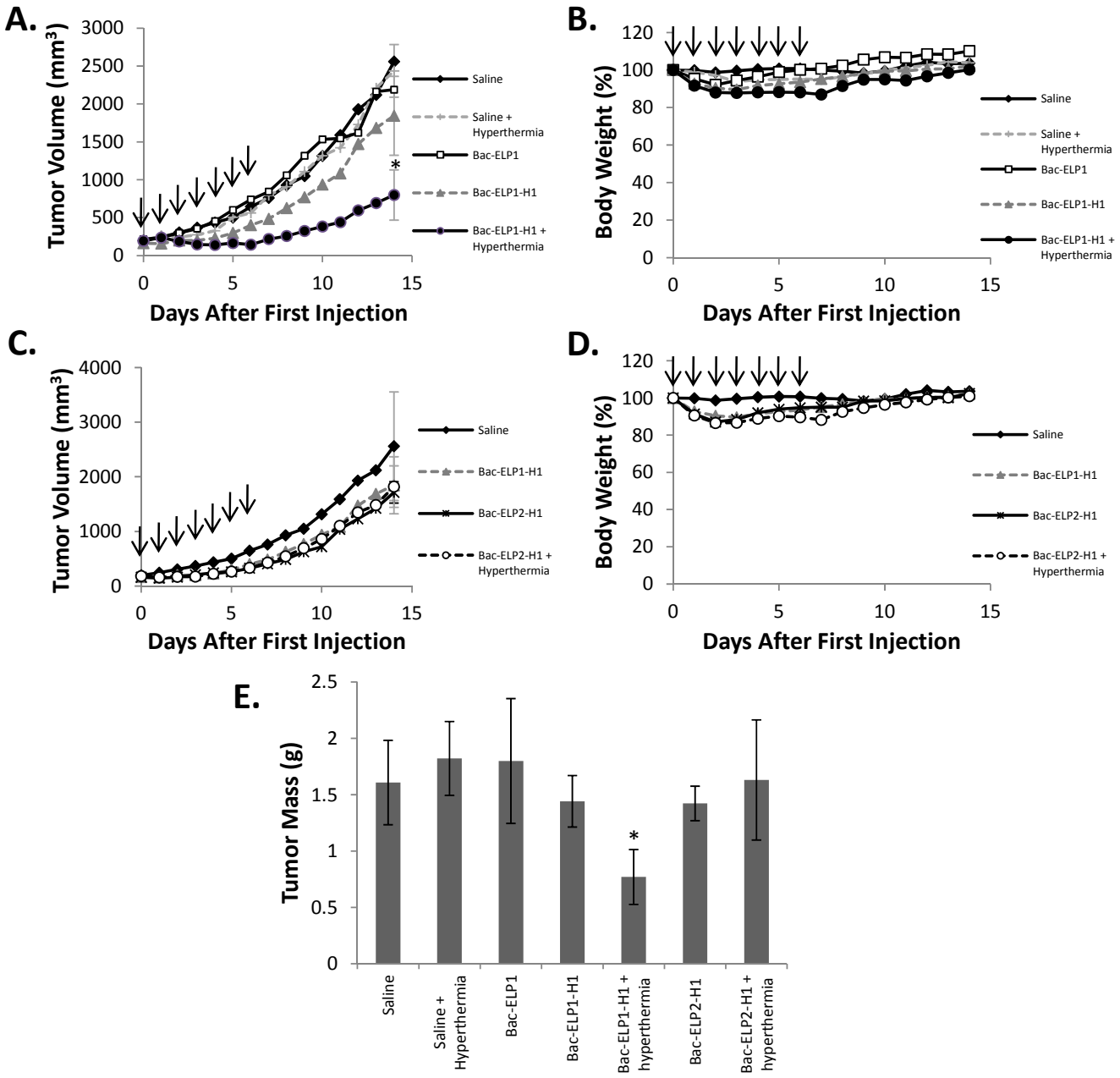



**Figure 7. Tumor Volume Reduction by Bac-ELP-H1.** E0771 Tumor volume (**A** and **C**) and corresponding body weight (**B** and **D**) after 7 daily treatments (arrows) with saline, saline + hyperthermia, Bac-ELP1, Bac-ELP1-H1, Bac-ELP1-H1 + hyperthermia (**A**,**B**), Bac-ELP2-H1, and Bac-ELP2-H1 + hyperthermia (**C**,**D**). Data represent the mean ± s.d. of 6 mice / group. *, Tumor volumes are statistically significant from control (one-way ANOVA, p = 0.0017, post hoc Bonferroni, 95% CI). **E.** Tumor mass on day 14. *, Tumor mass is statistically significant from control (one-way ANOVA, p = 0.0008, post hoc Bonferroni, 95% CI).

**Conclusion.**

Previous reports have described the characterization of an ELP-delivered c-Myc inhibitory peptide, including demonstration of its ability to inhibit the c-Myc pathway directly (1), its ability to sensitize cells to the actions of topoismerase II inhibitors (8), and its directed intracellular targeting via the use of carefully chosen CPPs (11). This work demonstrates that the ELP-delivered therapeutic peptide can be targeted to the site of a solid tumor *in vivo* using focused hyperthermia, and the polypeptide construct can escape the vasculature, enter the tumor cells, and inhibit their proliferation. Combined with previous data that the polypeptide can enhance the effects of traditional cytotoxic chemotherapeutics such as topoisomerase II inhibitors (8), this work suggests that the c-Myc inhibitory polypeptide could be a valuable addition to combination regiments for

adjuvant breast cancer therapy. Thermal targeting of Bac-ELP-H1 could be utilized for breast cancer therapy by treatment of tumor beds after resection of the primary tumor in order to prevent recurrence, for treatment of non-operable and possibly drug resistant recurrences, or for treatment of large metastases. This approach is advantageous because 1) the polypeptide is a macromolecule, and it will passively accumulate in tumor tissue due to the enhanced permeability and retention effect (12), 2) the thermally responsive nature makes active targeting of the polypeptide and its payload possible using focused hyperthermia, 3) the peptide inhibitor is specific to c-Myc, making this agent specific to tumor cells overexpressing the target protein, and 4) the polypeptide carrier is genetically encoded, which facilitates the addition of functional peptides and simplifies the purification process. These results demonstrate that ELP is an inert, biodegradable polymer capable of delivering a therapeutic peptide in a targeted, non-toxic manner. Given that the technology for administration of focused hyperthermia is already in place in the clinical setting in the form of MRI-guided high intensity focused ultrasound (13-16), the use of ELP as a vector has great potential to convert promising peptide agonists/antagonists into viable pharmaceutical agents.

**Key Research Accomplishments**

- Specific Aim 1: Plasma Clearance and Biodistribution of ELP – fused c-Myc Inhibitory Polypeptides.
    - Performed plasma clearance and tumor and organ distribution of polypeptide labeled with rhodamine in the E0771 syngeneic, orthotopic model using both IV and IP administration.
        - Found that the polypeptide exhibited a long plasma half – life, and that the IP route was a viable option for polypeptide delivery.
        - Demonstrated a 1.6 fold enhancement of tumor uptake of the polypeptide with hyperthermia treatment following IP administration.
        - Defined the organ biodistribution of Bac-ELP1-H1 after IV and IP administration.
    - Performed real-time imaging of tumors *in vivo* following polypeptide injection.
        - This analysis allows collection of multiple time points in the same animal, and was used to augment the detailed acute biodistribution study. We analyzed Bac-ELP1-H1 with and without tumor hyperthermia. We determined that polypeptide levels peak in tumors 6 h after IV injection, and tumor hyperthermia enhanced polypeptide deposition up to 3 fold.
- Specific Aim 2: Breast Tumor Reduction using CPP-ELP-H1 polypeptides.
    - Performed tumor reduction studies using Bac-ELP1-H1 and controls (hyperthermia alone, Bac-ELP1, and Bac-ELP2-H1) injected daily, with and without hyperthermia in the E0771 tumor model.
        - Demonstrated a slowed progression of tumor volume with the active Bac-ELP1-H1 polypeptide, and significant enhancement of this tumor reduction (70% reduction in tumor volume) by combining polypeptide treatment with hyperthermia. Demonstrated no tumor reduction by the Bac-ELP1 control polypeptide, or by hyperthermia alone.
        - Saw no obvious side effects beyond mild weight loss during the first 2 – 3 days of polypeptide administration.
    - Future experiments will examine tumor reduction efficacy in mouse models of other types of breast cancer, including triple negative breast cancer.

**Reportable Outcomes**

The *in vivo* results shown in this report were published as an abstract and presented in poster form at the 2011 annual meeting of the American Association for Cancer Research (AACR) in April in Orlando, FL and at the 2011 Era of Hope Meeting in August in Orlando, FL. A manuscript describing these results was submitted to *Science Translational Medicine* on 8/29/2011, and is currently under consideration. Also, a review article describing the use of this technology for delivery of peptide therapeutics was published in *Advanced Drug Delivery Reviews* (Bidwell, G. L. 3rd and Raucher, D. Cell penetrating elastin-like polypeptides for therapeutic peptide delivery. *Advanced Drug Delivery Reviews*. May 15, 2010).

## Conclusions

During the third year of funding, we have completed the goals outlined in this award proposal. The biodistribution study has confirmed our hypothesis that the ELP phase transition could be exploited for tumor thermal targeting. Also, tumor reduction experiments have demonstrated that the polypeptides under development do have antitumor activity against ER+ breast tumors, and we have achieved impressive tumor reduction by thermally targeting the antiproliferative polypeptide to the tumor site. Future experiments, to be performed under a no cost extension, will determine whether this novel agent has activity against other classes of breast cancer, including triple negative disease. Once the proposed studies are completed, we will have the efficacy and toxicity data necessary for advancing this technology toward the next phase of clinical development.

## References


1. Bidwell GL, 3rd, Raucher D. Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy. Mol Cancer Ther. 2005;4:1076-85.
2. Urry DW, Luan C-H, Parker TM, Gowda DC, Prasad KU, Reid MC, et al. Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity. J Am Chem Soc. 1991;113:4346-8.
3. Draeger LJ, Mullen GP. Interaction of the bHLH-zip domain of c-Myc with H1-type peptides. Characterization of helicity in the H1 peptides by NMR. J Biol Chem. 1994;269:1785-93.
4. Giorello L, Clerico L, Pescarolo MP, Vikhanskaya F, Salmona M, Colella G, et al. Inhibition of cancer cell growth and c-Myc transcriptional activity by a c-Myc helix 1-type peptide fused to an internalization sequence. Cancer Res. 1998;58:3654-9.
5. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. Journal of Biological Chemistry. 1994;269:10444-50.
6. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem. 1997;272:16010-7.
7. Sadler K, Eom KD, Yang JL, Dimitrova Y, Tam JP. Translocating proline-rich peptides from the antimicrobial peptide bactenecin 7. Biochemistry. 2002;41:14150-7.
8. Bidwell GL, 3rd, Raucher D. Enhancing the antiproliferative effect of topoisomerase II inhibitors using a polypeptide inhibitor of c-Myc. Biochem Pharmacol. 2006;71:248-56.
9. Bidwell GL, Raucher D. Therapeutic peptides for cancer therapy. Part I - peptide inhibitors of signal transduction cascades. Expert Opin Drug Deliv. 2009.
10. Dreher MR, Liu W, Michelich CR, Dewhirst MW, Chilkoti A. Thermal cycling enhances the accumulation of a temperature-sensitive biopolymer in solid tumors. Cancer Res. 2007;67:4418-24.
11. Bidwell GL, 3rd, Davis AN, Raucher D. Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides. J Control Release. 2009;135:2-10.
12. Maeda H, Seymour LW, Miyamoto Y. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. Bioconjug Chem. 1992;3:351-62.
13. Dromi S, Frenkel V, Luk A, Traughber B, Angstadt M, Bur M, et al. Pulsed-high intensity focused ultrasound and low temperature-sensitive liposomes for enhanced targeted drug delivery and antitumor effect. Clin Cancer Res. 2007;13:2722-7.
14. Frenkel V, Etherington A, Greene M, Quijano J, Xie J, Hunter F, et al. Delivery of liposomal doxorubicin (Doxil) in a breast cancer tumor model: investigation of potential enhancement by pulsed-high intensity focused ultrasound exposure. Acad Radiol. 2006;13:469-79.
15. Hertzberg Y, Volovick A, Zur Y, Medan Y, Vitek S, Navon G. Ultrasound focusing using magnetic resonance acoustic radiation force imaging: application to ultrasound transcranial therapy. Med Phys. 2010;37:2934-42.
16. Leslie TA, Kennedy JE. High intensity focused ultrasound in the treatment of abdominal and gynaecological diseases. Int J Hyperthermia. 2007;23:173-82.